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Tanguy Lechertier, Valentina Sirri, Danièle Hernandez-Verdun, Pascal Roussel. A B23-interacting sequence as a tool to visualize protein interactions in a cellular context.. *Journal of Cell Science*, 2006, 120 (2), pp.265-275. 10.1242/jcs.03345 . hal-00122585

HAL Id: hal-00122585

<https://hal.science/hal-00122585>

Submitted on 22 Jul 2007

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A B23-interacting sequence as a tool to visualize protein interactions in a cellular context

Tanguy Lechertier, Valentina Sirri, Danièle Hernandez-Verdun and Pascal Roussel

Institut Jacques Monod, UMR 7592 CNRS/Universités Paris 6 et 7, 2 Place Jussieu, 75251 Paris Cedex 05, France

Number of words (without reference list): 7804

Running head: Nucleolar targeting

Keywords: nucleolus, protein B23, NoLS, targeting, protein interaction

Corresponding author : Danièle Hernandez-Verdun
 Institut Jacques Monod
 CNRS/Universités Paris 6 et 7
 2 place Jussieu
 F-75251 Paris cedex 05
 Phone: +33 1 44 27 40 38
 Fax: +33 1 44 27 59 94
 e-mail: dhernand@ccr.jussieu.fr

Summary

We report the characterization of a nucleolar localization sequence (NoLS) that targets the green fluorescent protein (GFP) into the granular component (GC) of nucleoli. This NoLS interacts in vitro specifically and directly with the major nucleolar protein B23 and more precisely with the region of B23 including the two acidic stretches. The affinity of NoLS for B23 is stronger than that of the HIV-1 Rev protein in vitro. Moreover, B23-NoLS interaction occurs also in vivo. Indeed, 1) NoLS confers on the GFP the behavior of B23 throughout the cell cycle, 2) the GFP-NoLS fusion and B23 remain colocalized after drug treatments, 3) a selective delocalization of B23 from nucleoli to nucleoplasm induces a concomitant delocalization of the GFP-NoLS fusion, and 4) the fusion of NoLS to fibrillarin makes it possible to colocalize fibrillarin and B23. Interestingly, by fusing NoLS to fibrillarin, both fibrillarin and the fibrillarin partner Nop56 are mislocalized in the GC of nucleoli. Similarly by fusing the NoLS to MafG, part of the nuclear transcription factor NF-E2 composed of both MafG and p45 NF-E2, NF-E2 is re-directed from the nucleoplasm to the nucleoli. Thus, we propose that NoLS may be used as a tool to visualize and prove protein interactions in a cellular context.

Introduction

The architecture of the nucleus is complex and the nuclear functions are precisely located in the nuclear volume. Morphologically distinct structures or nuclear bodies are present. They correspond to specialized domains implicated in essential processes such as transcription and splicing (Matera, 1999; Spector, 2001; Strouboulis and Wolffe, 1996), or represent in the case of the nucleolus the site of ribosome biogenesis (Mélèse and Xue, 1995). This complicated architecture was proposed to be linked to the dynamics of proteins and RNP complexes in the nucleus (Misteli, 2001; Phair and Misteli, 2000). In the nucleoplasm, the high mobility of proteins would ensure their diffusion and availability throughout the nucleus. In nuclear bodies the mobility of proteins is reduced most probably by interactions with partners. This determines a residence time based on affinities and functions of the proteins (Chen and Huang, 2001; Dundr et al., 2004; Snaar et al., 2000). If this model based on analyses of the nuclear traffic in living cells is correct, protein interactions should govern their nuclear distribution. By modifying the normal affinity of proteins it might be possible to create experimental interactions that would provide information and permit analysis of the model.

The nucleolus is the paradigm of functional compartmentation in the nucleus (Strouboulis and Wolffe, 1996). Ribosomal RNAs (rRNAs) are synthesized, processed and assembled with ribosomal proteins to form the small 40S and large 60S pre-ribosome subunits in the nucleolus (for a review, see Fatica and Tollervy, 2002; Gébrane-Younès et al., 2005; Hernandez-Verdun, 2006; Shaw and Jordan, 1995). The dynamic integration of these processes generates a typical nucleolar organization revealing the compartmentation of the different steps. Three main specific components are observed by electron microscopy (Scheer and Hock, 1999), the fibrillar centers (FCs), the dense fibrillar component (DFC), and the granular component (GC). In the active nucleolus, the early rRNA processing proteins are

associated with transcripts during elongation and localized in the internal part of the nucleolus, i.e. in the DFC. The processing proteins associated with late steps of rRNA processing are localized in the external region of the nucleolus, i.e. in the GC.

Protein B23 (NPM, nucleophosmin, numatrin or NO38) is an abundant phosphoprotein localized mainly in the nucleoli during interphase, at the chromosome periphery during mitosis, and in foci called prenucleolar bodies (PNBs) at the exit from mitosis. It is a multifunctional protein that has nucleic acid binding, ribonuclease and molecular chaperone activities. By mapping the functional domains of B23, it was found that the N-terminal domain of B23 is necessary and sufficient to bind nucleic acids, the molecular chaperone activity is contained in the N-terminal half of the protein, and the central region of the molecule is required for ribonuclease activity (Hingorani et al., 2000; Szebeni et al., 2003). The C-terminal region of B23 is preferentially associated with 28S pre-rRNA and its ribonuclease activity processes the internal transcribed spacer 2 of the pre-rRNA (Huang et al., 2005; Itahana et al., 2003; Savkur and Olson, 1998). The chaperone activity of B23 is controlled by protein kinase CK2 and is most probably linked to the ability of B23 to form large oligomers in native conditions (Herrera et al., 1996; Szebeni et al., 2003). In addition, B23 interacts with other nucleolar proteins such as nucleolin (Li et al., 1996), protein p120 (Valdez et al., 1994), ARF (Korgaonkar et al., 2005) and the HIV-1 Rev protein (Fankhauser et al., 1991). In this latter case B23 could serve as molecular chaperone for Rev (Olson, 2004). In addition, it was also proposed that B23 could play a role in nucleocytoplasmic transport since it can shuttle between nucleus and cytoplasm (Borer et al., 1989; Fankhauser et al., 1991). B23 is phosphorylated before mitosis and this modification reduces RNA binding by B23 (Okuwaki et al., 2002). This suggests that phosphorylation at the beginning of mitosis may promote B23 translocation from nucleoli to chromosome periphery. Interestingly, mutated B23 genes were found in a number of cancers and their implication in haematological

disorders was demonstrated (Grisendi et al., 2005). B23 therefore appears to be a key protein in ribosome biogenesis and cell cycle.

In the case of nucleolar targeting of viral proteins, it was proposed that a nucleolar sequence signal (NoLS) could be involved (Dang and Lee, 1989; Kubota et al., 1989; Siomi et al., 1988). This is not a particularity of viral proteins since similar sequences were also found in several endogenous nucleolar proteins (Dang and Lee, 1989; Korgaonkar et al., 2005; Song and Wu, 2005; Valdez et al., 1994; Weber et al., 2000). In the present study, we have characterized a novel protein sequence that targets the green fluorescent protein (GFP) to the GC of nucleoli. This sequence designated NoLS interacts both *in vitro* and *in vivo* with B23. In addition, we show that on one hand, by fusing the NoLS to fibrillarin, both fibrillarin and Nop56, another member of the core proteins of the box C/D snoRNP complexes, are relocalized from the DFC to the GC of nucleoli. On the other hand, by fusing the NoLS to MafG, a subunit of the transcription factor NF-E2 composed of MafG and p45 NF-E2 (Tramier et al., 2002), NF-E2 is re-directed from the nucleoplasm to the GC of the nucleoli. Thus, we demonstrate here the capability to relocalize nuclear complexes by adding an affinity for B23 to one subunit of the complex and therefore, we propose that such a B23-interacting sequence may be used as tool to visualize and to prove protein interactions in a cellular context by fluorescence microscope observations.

Results

A novel nucleolar localization sequence

During PCR-amplification of the DNA sequence encoding human Sirtuin 1, a mutant was generated which showed a nucleolar localization when expressed fused to GFP in HeLa cells. The analysis of the DNA sequence of this mutant revealed a misreading of the DNA polymerase having led to the loss of four nucleotides (nt 262-265) of the Sirtuin 1-coding DNA sequence and consequently, to a change in reading frame (Fig. 1A). Therefore, the resulting mutant protein corresponds to the first 87 amino acids of Sirtuin 1 followed by a “synthetic” sequence of 93 amino acids corresponding to the Sirtuin 1 DNA sequence (nt 266-544). By subcloning both parts of this mutant protein separately into a GFP-fusion plasmid, we observed that the first 87 amino acids of Sirtuin 1 (Δ Sirtuin 1) expressed fused to the GFP in HeLa cells did not show any obvious nucleolar localization contrary to the “synthetic” sequence which was able to address the GFP to the nucleolus. The new sequence (Fig. 1A,B) was therefore designated NoLS. This novel NoLS possesses no significant homology with the NoLSs previously described except for the presence of a stretch of basic residues (Fig. 1B, in red) as already reported for other nucleolus-localizing proteins.

To delineate this NoLS, different constructs were generated (Fig. 1C) and expressed in HeLa cells as GFP fused proteins (Fig. 1D). Undoubtedly, the stretch of basic residues (in red Fig. 1B,C) is strongly implicated in nucleolar localization. Indeed, only the constructs NoLS, NoLS/33-93, NoLS/31-63 and NoLS/1-50 which possessed the stretch of basic residues were localized mostly if not exclusively in nucleoli, whereas the other constructs were not (NoLS/51-93) or only slightly (NoLS/1-32) enriched in nucleoli (Fig. 1D). However, the entire sequence of NoLS seems to be implicated in nucleolar localization. On one hand, the construct NoLS/1-32 was also slightly enriched in nucleoli and on the other hand, even if the

construct NoLS/51-93 did not exhibit nucleolar localization (Fig. 1Dk), the C-terminal region of NoLS appeared to facilitate targeting of the GFP to the nucleolus as observed by comparing the localization of NoLS/33-93 to that of NoLS/31-63 (Fig. 1Dc and Di). Both constructs were localized in nucleoli but in the absence of the C-terminal end of NoLS (Fig. 1Di), a certain level of nucleoplasmic labeling was also observed contrary to what occurred in the presence of the C-terminal end (Fig. 1Dc). For these reasons, the complete NoLS was used for all subsequent experiments.

The novel NoLS interacts specifically and directly with B23 in vitro

To further understand how GFP-NoLS localized specifically in nucleoli and since previous studies had shown that several NoLSs interact with the major nucleolar protein B23 in vitro, we examined by glutathione S-transferase (GST) pull down experiments the interaction between B23 and the novel NoLS (Fig. 2A). For this purpose, a GST-NoLS fusion was generated and pull down assays were carried out on whole cell extracts. As shown in Fig. 2A, analysis by Western blot of the bound proteins revealed that B23 interacts with the NoLS contrary to other nucleolar proteins such as Nopp140, nucleolin the other major nucleolar protein, and Nop52. The interaction between B23 and the NoLS is direct as demonstrated by GST-NoLS pull down assays carried out on purified B23 (Fig. 2B). To determine the region within B23 required for B23-NoLS interaction, various B23 truncation mutants were generated (Fig. 2C) and expressed in HeLa cells as GFP or DsRed fused proteins. GST-NoLS pull down were then performed to identify which B23 mutants interacted with NoLS. The results showed that the central region, i.e. the region including the two acidic stretches of B23 (amino acids 120 to 132 and 161 to 188), governs interaction between B23 and NoLS whereas neither the N-terminal (amino acids 1 to 119) nor the C-terminal (amino acids 187-294) region participates in this interaction. In addition, a comparison between the results obtained

with the mutants B23/1-119, B23/1-132, B23/1-160 and B23/1-186, i.e. the B23 mutants possessing the B23 residues 1-119, 1-132, 1-160 and 1-186, respectively, indicates that the first acidic stretch of B23 (amino acids 120 to 132) is sufficient to promote B23-NoLS interaction.

The novel NoLS has a stronger affinity for B23 than for HIV-1 Rev in vitro

To evaluate the strength of the B23-NoLS interaction, we compared NoLS to the HIV-1 Rev protein, previously identified as a B23-interacting protein localized in the nucleolus. For this purpose, the same amounts of GST, GST-NoLS and GST-Rev were bound to sepharose beads and incubated for increasing times with the same whole HeLa cell extracts. After the GST pull down assays, the bound proteins were resuspended in SDS-PAGE sample buffer, electrophoresed and analyzed by coomassie blue staining to check that no significant loss of GST, GST-NoLS and/or GST-Rev had occurred during the experiments. In parallel, the same samples were electrophoresed, Western-blotted onto nitrocellulose membranes and B23 was detected and quantified. As illustrated for a representative experiment (Fig. 3A,B), B23 interacts with both NoLS and Rev but the B23-NoLS interaction occurred more rapidly and the amount of bound B23 was higher than in the B23-Rev interaction. Indeed, the comparison of the amounts of B23 bound to NoLS and to Rev (Fig. 3B) reveals that the amount of B23 bound to NoLS was 4 times greater than that bound to Rev after 1.5 hours, 3 times after 3 hours and 2.5 times after 20 hours of incubation. The fact that the NoLS exhibits a strong affinity for B23 in vitro prompted us to verify the B23-NoLS interaction in vivo.

The novel NoLS interacts specifically with protein B23 in vivo

To precisely locate NoLS in nucleoli and to determine whether this localization is compatible with B23-NoLS interaction in vivo, we localized the GFP-NoLS fusion together with

endogenous fibrillarin and B23 in interphasic HeLa cells. As shown Fig. 4A, fibrillarin known to be localized in the DFC of nucleoli did not colocalize with GFP-NoLS. Conversely B23, mostly located in the GC of nucleoli, appeared to strictly colocalize with GFP-NoLS indicating that GFP-NoLS is mostly localized in the GC of nucleoli. In addition, this colocalization is in agreement with the fact that interaction between B23 and NoLS occurs *in vivo*. Concerning mitosis, the behavior of GFP-NoLS appeared to be similar to that of B23 (Fig. 4B). Indeed, as is well known for B23, GFP-NoLS was enriched at the periphery of chromosomes during metaphase (Fig. 4Ba-d) and recruited in PNBs at telophase (Fig. 4Be-h) before being relocated in new nucleoli formed in early G1 (Fig. 4Bi-l). Therefore the NoLS confers on GFP a localization similar to that of B23 throughout the cell cycle.

The localization of NoLS during the cell cycle is compatible with B23-NoLS interaction *in vivo*. This is reinforced by the fact that the colocalization of both NoLS and B23 was maintained when HeLa cells were treated with drugs inducing a dramatic reorganization of the nucleolus (Fig. 5A) and hence relocation of nucleolar proteins. Indeed after actinomycin D treatment that inhibits rDNA transcription and consequently induces nucleolar reorganization (Hadjiolov, 1985), NoLS is colocalized with B23 (Fig. 5Aa-d) and excluded from caps where fibrillarin is located (Fig. 5Ae-h). Similarly, after treatment with the cyclin-dependent kinase (CDK) inhibitor roscovitine that also induces dramatic disorganization of the nucleolus (Sirri et al., 2002), NoLS is still observed colocalized with B23 (Fig. 5Ai-l) but not with fibrillarin (Fig. 5Am-p).

To prove B23-NoLS interaction *in vivo*, a human B23 mutant, designated mutant A by Falini and coworkers (Falini et al., 2005) was used to selectively exclude B23 from the nucleolus and to follow the localization of NoLS. This B23 mutant corresponds to a substitution of 11 novel amino acids for the seven C-terminal residues of the wild type B23 generating a consensus nuclear export signal (NES). This B23-NES mutant localizes in the

cytoplasm of interphasic NIH-3T3 cells when expressed fused to GFP in a Crm1-dependent manner (Falini et al., 2006). As previously reported (Falini et al., 2006), in the presence of the Crm1-inhibitor leptomycin B, B23-NES relocates in the nucleus and is mostly excluded from the nucleoli (Fig. 5Ba,d). Interestingly, most probably due to the capability of B23 to multimerize, after leptomycin B treatment the endogenous B23 is delocated from nucleoli in NIH-3T3 cells that express B23-NES, whereas the endogenous B23 remains localized in nucleoli in cells which do not express B23-NES after the same treatment (Fig. 5Ba-c). Similarly in NIH-3T3 cells expressing B23-NES and treated with leptomycin B, most of NoLS leaves the nucleoli and localizes in the nucleoplasm (Fig. 5Bd-f). Since the selective delocalization of the endogenous B23 from nucleoli to nucleoplasm similarly modifies the localization of NoLS, we conclude that B23-NoLS interaction occurs *in vivo*. Consequently, we propose that fusing the NoLS to GFP confers on GFP a high affinity for B23. This affinity is maintained throughout the cell cycle, and therefore the GFP-NoLS fusion is able to interact specifically with B23 *in vivo* and to adopt the behavior of B23 during interphase as well as during mitosis.

The novel NoLS may be used as a new tool to visualize protein interactions in the cell nucleolus

When fused to GFP, the novel NoLS is able to target GFP to the nucleolus by conferring on the GFP a high affinity for the nucleolar protein B23. However, it may be assumed that GFP does not possess any particular affinity in eukaryotic cells and therefore that modifying its localization is easier than for physiological proteins. Consequently, it may be asked whether addition of the NoLS to a physiological nucleolar protein could modify the localization of this protein. To answer this question, we localized both fibrillarin-GFP fusion and fibrillarin-NoLS-GFP fusion together with B23 (expressed as DsRed-B23 fusion) in interphasic HeLa

cells. As shown Fig. 6a-d, fibrillarin known to be localized in the DFC of nucleoli was not observed colocalized with B23 known to be mostly located in the GC of nucleoli. Conversely fibrillarin-NoLS appeared to strictly colocalize with B23 (Fig. 6e-h) indicating that the addition of the NoLS to fibrillarin makes it possible to relocate fibrillarin from the DFC to the GC of nucleoli, i.e. to confer on fibrillarin the same localization as B23.

It may also be asked whether the addition of NoLS to one subunit of a nucleolar protein complex could modify the localization of the entire complex. In this case, NoLS might be used as tool to visualize and to prove protein interactions in the nucleolus by adding NoLS to one subunit of a nucleolar protein complex and determining whether the other subunits of the complex are mislocalized. To address this question, we checked the effect of the mislocalization of fibrillarin in the GC of nucleoli, i.e. when expressed as fibrillarin-NoLS, on the localization of Nop56, another member of the core proteins of the box C/D snoRNP complexes. We first verified that both fibrillarin and the fibrillarin partner Nop56 were colocalized in the DFC of nucleoli when coexpressed in interphasic HeLa cells as fibrillarin-GFP and DsRed-Nop56 fusions (Fig. 6i-l). The colocalization of both proteins was maintained when fibrillarin-NoLS and Nop56 were coexpressed in interphasic HeLa cells (Fig. 6m-p) showing that the mislocalization of fibrillarin in the GC of nucleoli induces the mislocalization of its partner Nop56. The relocation of Nop56 into the GC of nucleoli when it was coexpressed with fibrillarin-NoLS was clearly due to the interaction between Nop56 and fibrillarin. Indeed no relocation was observed for Nopp140, a protein that is not a member of the core proteins of the box C/D snoRNP complexes. As well established, fibrillarin and Nopp140 were colocalized in the DFC of nucleoli in interphasic HeLa cells (Fig. 6q-t), but this colocalization was no longer maintained when fibrillarin delocalized into the GC of nucleoli (Fig. 6u-x). In addition, the fact that the localization of Nopp140 was not modified in cells expressing fibrillarin-NoLS also indicated that the nucleolar morphology

was maintained. The expression of fibrillarin-NoLS did not induce disappearance of DFC and consequently a general delocalization of proteins of the DFC.

The novel NoLS may be used as a new tool to visualize protein interactions in the cell nucleus

When fused to the fibrillarin, NoLS is able to delocalize fibrillarin from the DFC to the GC of nucleoli by conferring on fibrillarin high affinity for B23. Moreover, the addition of NoLS to one subunit of a protein complex could modify the nucleolar localization of the complex. Consequently, it may be asked whether addition of NoLS to a nuclear protein could modify the localization of the protein and target it to the nucleolus and whether the addition of the NoLS to one subunit of a nuclear protein complex could target the entire complex to the nucleolus. Thus, the NoLS might be used as tool to visualize and to prove protein interactions in the nucleus.

To answer these questions, the transcription factor NF-E2 composed of two subunits, MafG and p45 NF-E2, was used as model of a heterodimeric nuclear protein. These two protein subunits were produced as GFP-P45 and DsRed-MafG fusions and NoLS was added to the amino-terminus of MafG to generate the DsRed-NoLS-MafG fusion. We first verified that the GFP-P45 and DsRed-MafG fusions exhibited the expected localization when expressed in HeLa cells. Indeed, as previously reported (Tramier et al., 2002), both proteins are localized in the nucleus and are largely excluded from nucleoli as observed by comparing their position to that of B23 (Fig. 7a-d,e-h). More interestingly, the DsRed-NoLS-MafG fusion localized in nucleoli and the coexpression of both DsRed-NoLS-MafG and GFP-B23 showed a strict colocalization of both proteins (Fig. 7i-l). Therefore, NoLS made it possible to modify the localization of MafG and to target it to nucleoli. The capability of NoLS to relocate a nuclear protein to the nucleoli and more precisely to the GC of the nucleoli where

B23 is located, was also verified for CDK9 (our unpublished data).

Concerning the localization of the MafG partner P45, coexpression of both DsRed-NoLS-MafG and GFP-P45 in HeLa cells showed that the relocalization of MafG in nucleoli led to the relocalization of most P45 to the same sites (Fig. 7m-p) and thus proved the interaction of these two proteins when they are coexpressed in HeLa cells. Moreover, the relocalization of P45 to nucleoli when coexpressed with NoLS-MafG was clearly due to the interaction between P45 and MafG. Indeed, the expression of DsRed-NoLS in HeLa cells expressing GFP-P45 did not modify the localization of GFP-P45 (Fig. 7q-t). In addition, coexpression of GFP-Sirtuin 1, a nuclear protein that possesses no affinity for MafG, with DsRed-NoLS-MafG did not change the localization of the former protein (Fig. 7u-x). In conclusion, we propose that adding the NoLS to one subunit of a nuclear protein complex makes it possible to target the entire complex to the nucleolus. Consequently, such an approach might be used to demonstrate protein interactions in a cellular context.

Discussion

A novel NoLS targets GFP to the nucleolus and interacts with B23

In this study, we generated a protein sequence which confers to GFP a specific nucleolar localization. This “synthetic” NoLS possesses no significant homology with the NoLSs previously described except for the presence of a stretch of basic residues as already reported for viral nucleolus-localizing proteins such as HIV-1 Rev (Kubota et al., 1989), HIV-1 Tat (Dang and Lee, 1989) and HTLV-1 Rex (Siomi et al., 1988) and for cellular proteins such as the nucleolar protein p120 (Valdez et al., 1994), Survivin-deltaEx3 (Song and Wu, 2005) and HSP70 (Dang and Lee, 1989). As demonstrated by the localization of deletion mutants of this novel NoLS, the stretch of basic residues plays a key role in nucleolar localization. Interestingly, as observed here, the capability of numerous proteins to adopt a nucleolar localization is correlated with their interaction with B23 (Adachi et al., 1993; Fankhauser et al., 1991; Korgaonkar et al., 2005; Li, 1997; Valdez et al., 1994). Because of the ability of numerous nucleolar proteins to interact with B23 and because this major nucleolar protein shuttles constantly between nucleus and cytoplasm (Borer et al., 1989), it was often suggested that B23 might be a transporter for nucleolar proteins possessing a NoLS (Fankhauser et al., 1991; Li, 1997; Valdez et al., 1994). Even if this tempting hypothesis has never been demonstrated, recent results obtained on stable U2OS-derived cell lines with reduced B23 expression levels showed that the nucleolar localization of ARF is linked to B23 (Korgaonkar et al., 2005). Indeed, the reduced expression of B23 induced a partial delocalization of ARF from nucleoli to nucleoplasm. The authors therefore concluded that B23 targets ARF to nucleoli in a dose-dependent manner. Nevertheless, this result does not make it possible to discriminate between a role of B23 in the transport of ARF from nucleoplasm to nucleoli and/or a role of B23 in the retention of ARF in nucleoli.

However, contrary to what had been suggested by several authors (Fankhauser et al., 1991; Li, 1997; Valdez et al., 1994), a role of B23 in the transport of proteins to nucleoli does not seem mandatory. In fact, the recent analysis of the intranuclear dynamics of proteins in living cells has shown that nuclear proteins can diffuse within the nucleoplasm (Phair and Misteli, 2000; Sprague and McNally, 2005). Concerning the nucleolus, it was demonstrated that nucleolar proteins rapidly associate with and dissociate from nucleolar components in a continuous exchange with the nucleoplasm (Chen and Huang, 2001; Dundr et al., 2004; Phair and Misteli, 2000; Snaar et al., 2000). The authors suggest the existence of compartment-specific retention mechanisms for proteins in nuclear bodies implying that the residence time of a particular molecule in a given nuclear body depends on its specific interactions (Misteli, 2001). Consequently, protein interactions should govern the nuclear distribution of proteins and a NoLS might actually be a nucleolar molecule-interacting sequence.

A novel NoLS or a new B23-interacting sequence

The NoLS described here interacts with the central region of B23, i.e. the region including the two acidic stretches (amino acids 120 to 132 and 161 to 188) as already reported for HTLV-1 Rex (Adachi et al., 1993). B23-NoLS interaction is direct and specific as demonstrated by the fact that the other nucleolar proteins tested showed no affinity for this novel NoLS. However, it seems unlikely that B23-NoLS interaction is only electrostatic occurring between the acidic stretches of B23 and the cluster of basic residues in NoLS. Indeed the other major nucleolar protein, nucleolin, that possesses four stretches of acidic residues in its N-terminal region does not interact with NoLS. Whatever type of interaction exists between B23 and NoLS, the affinity of NoLS for B23 in vitro was stronger than that of the HIV-1 Rev protein for B23. Therefore, NoLS possesses a strong affinity for B23 in vitro and also in vivo as observed by the colocalization of the GFP-NoLS fusion and B23 in the GC of nucleoli during interphase,

and by the fact that NoLS confers to GFP the behavior of B23 during mitosis. In addition, GFP-NoLS fusion and B23 remain colocalized after actinomycin D or roscovitine treatment. Furthermore, a selective delocalization of B23 from nucleoli to nucleoplasm induces a concomitant delocalization of the GFP-NoLS fusion from nucleoli to nucleoplasm because of B23-NoLS interaction. The behavior of GFP-NoLS during mitosis, i.e. mainly located at the periphery of chromosomes and recruited in PNBs in telophase, shows that rather than a sequence that targets the GFP to nucleoli, NoLS appears to be a new B23-interacting sequence, i.e. a sequence interacting with the major nucleolar protein. It is interesting to note that B23-NoLS interaction occurs throughout the cell cycle and therefore does not seem to be influenced by the post-translational modifications of B23. The observation that NoLS is a B23-interacting sequence *in vivo* is reinforced by the capacity of NoLS to relocate fibrillarin from the DFC to the GC of nucleoli where B23 is localized. By providing fibrillarin with an additional affinity for B23, the localization of fibrillarin within the nucleolus is modified.

The new B23-interacting sequence may be used as a new tool to visualize protein interactions in a cellular context

The proteomic approaches currently developed show us how necessary it is to understand the relationship between proteins, and in particular between nuclear proteins. Generally, protein interactions are studied by *in vitro* experiments. This implies that it is not possible to analyze interactions based on cellular activities. To date, the most widely used approaches making it possible to analyze protein interactions in a cellular context are FRET (Selvin, 2000; Wouters et al., 2001) and bimolecular fluorescence complementation (BiFC) (Hu et al., 2002). However, with both these methods only direct interactions are accessible.

Because conferring to a protein an additional strong affinity for B23 makes it possible to sequester and/or relocate this protein to or within the nucleolus as verified for GFP, fibrillarin, MafG (this study) and CDK9 (our unpublished data), we hypothesized that such a approach might be used to relocate a protein complex to or within the nucleolus. This hypothesis was proved correct by analyzing on one hand, two members of the core proteins of the box C/D snoRNP complexes, namely fibrillarin and Nop56, and on the other hand, the MafG-P45 complex. This latter complex was chosen because a previous study carried out by FRET demonstrated MafG-P45 interaction when both proteins are expressed in HeLa cells (Tramier et al., 2002). Here, we show that the delocalization of fibrillarin from the DFC to the GC of nucleoli by addition of NoLS results in the co-delocalization of Nop56 from the DFC to the GC of nucleoli. Similarly the delocalization of MafG from nucleoplasm to nucleoli by addition of NoLS results in the co-delocalization of P45 to the nucleoli. This co-delocalization is only observed for interacting proteins and does not occur for the fibrillarin and Nopp140 pair as expected since Nopp140 is not an integral component of the box C/D snoRNP complexes (Yang et al., 2000); likewise it does not occur for proteins without affinity for each other such as MafG and Sirtuin 1. Therefore, the co-delocalization of two proteins demonstrates a strong interaction between these two proteins in a cellular context. Consequently, this approach based on the targeting of a complex to the GC of nucleoli might be used to determine whether a given protein interaction occurring in vitro also occurs in vivo and/or to identify the partner(s) of a specific protein in a cellular context. It is noteworthy that this new and simple approach for in vivo binding studies is most probably made possible by the fact that 1) NoLS possesses a strong affinity for B23, i.e. the major nucleolar protein, and 2) B23-NoLS interaction does not seem to be influenced by the post-translational modifications of B23. Fusing NoLS to a nuclear protein results in the delocalization of this protein into the GC of nucleoli, a phenomenon easily assessed by fluorescence microscopy.

This new tool aimed at analyzing protein interactions offers the advantage of assessing direct protein interactions in a cellular context as well as indirect interactions. Indeed, contrary to FRET and BiFC based on direct interaction between two proteins, this new approach is based on the targeting to nucleoli of a protein together with its directly or indirectly associated partners. In addition, when antibodies are available, direct or indirect interactions between proteins may be tested by expressing the first protein fused to NoLS and analyzing by immunofluorescence the possible delocalization to nucleoli of its potential partners. Moreover, by varying experimental conditions, this simple approach should make it possible to elucidate protein interactions but also to uncover the mechanisms that regulate such interactions. In conclusion, this new approach should lead to a better integrated vision of the cell nucleus.

Acknowledgments

The authors are grateful to N. Bolli, B. Falini, F. Ishikawa, E. Le Rouzic, U. T. Meier, V. Mignotte, M.O.J. Olson and R.Y. Tsien for generously providing constructs or antibodies and to A.L. Haenni for critical reading of the manuscript. T.L. is recipient of an undergraduate fellowship from Ministère de l'Education Nationale, de la Recherche et de la Technologie. This study was supported in part by grants from the Centre National de la Recherche Scientifique and the Association pour la Recherche sur le Cancer (Contract 3303).

Materials and Methods

Cell culture, transfection, immunofluorescence

HeLa cells were cultured in MEM with GlutaMAX™ (Invitrogen) containing 10% FCS and NIH-3T3 cells in DMEM (Invitrogen) containing 10% FCS and 2 mM L-glutamine (Invitrogen) in a 5% CO₂ incubator at 37°C. Cells were transfected with purified plasmids using effectene (Qiagen) according to the manufacture's instructions. For synchronization in mitosis, HeLa cells were accumulated in prometaphase by nocodazole treatment (0.04 µg/ml for 3 hours), selectively harvested by mechanical shock, washed, and resuspended in nocodazole-free medium for 30 or 60 minutes. The drugs used were leptomycin B (Calbiochem) at 20 nM for 4 hours, roscovitine (Calbiochem) at 75 µM for 2 hours and actinomycin D (Sigma) at 0.1 µg/ml for 2 hours. For immunofluorescence, cells were plated on a coverslip in 6-well plates (Techno Plastic Products AG) and transfected 24 hours later. At 16-24 hours post transfection, the cells were fixed in 2% paraformaldehyde for 20 minutes at room temperature, permeabilized with 0.5% Triton X-100 for 5 minutes and prepared for fluorescence microscopy as previously described (Sirri et al., 2002). Alternatively, the cells were fixed with 100% methanol for 10 minutes at -20°C, dried and rinsed several times with PBS before being incubated with antibodies. The antibodies used were goat polyclonal anti-B23 (Santa Cruz Biotechnology), mouse monoclonal anti-B23 (Sigma), a previously characterized human autoimmune serum with specificity against fibrillarin (O61; Sirri et al., 2002) and Texas Red-conjugated secondary antibodies specific for goat, mouse and human IgGs (Jackson ImmunoResearch Laboratories). Samples were incubated with 4,6-diamidino-2-phenylindole (DAPI) to visualize DNA and mounted with the antifading solution AF1 (Citifluor). Fluorescence microscopy was performed using a CCD camera Leitz DMRB and images assembled using Adobe Photoshop.

Plasmid constructs

Entire or partial DNA sequences encoding the NoLS were amplified by PCR with specific primers inserting the restriction sites EcoRI and BamHI respectively in the 5' and 3' ends of the PCR products. Partial DNA sequences of NoLS were cloned into the EcoRI/BamHI sites of pEGFP-C2 (Clontech) and the entire NoLS sequence into the EcoRI/BamHI sites of both pEGFP-C2 and pDsRed-m-C1 (Clontech). The same strategy was used to clone NoLS and B23 into the BamHI/EcoRI sites of the pGEX4T1 (Amersham) to generate the GST-NoLS and GST-B23 constructs. GST-Rev was obtained by subcloning the DNA sequence encoding HIV-1 Rev from pAS1B-Rev (kindly provided by E. Le Rouzic, Institut Cochin, Paris, France) into the EcoRI/XhoI sites of pGEX4T1. Full-length and B23 mutants were prepared by PCR-amplification from full-length human cDNA (clone IMAGE 5575414) and subcloning into the EcoRI/BamHI sites of pEGFP-C1 (Clontech) for full-length B23 and mutant B23/121-186, or of pDsRed-m-C1 for full-length B23 and mutants B23/1-119, B23/1-132, B23/1-160, B23/1-186 and B23/187-294. GFP-B23-NES was kindly provided by B. Falini and N. Bolli (University of Perugia, Perugia, Italy) and corresponds to pEGFP-C1-NPMmA (Falini et al., 2005). Fibrillarin-GFP and fibrillarin-RFP were obtained by PCR-amplification from human cDNA and subcloning into the EcoRI/BamHI sites of pEGFP-N1 (Clontech) and pmRFP-N1 kindly provided by R.Y. Tsien (University of California, San Diego, USA). Fibrillarin cDNA and the DNA sequence of NoLS were fused by the "gene soeing" strategy and the PCR product subcloned into the EcoRI/BamHI sites of pEGFP-N1 and pmRFP-N1 to obtain respectively fibrillarin-NoLS-GFP and fibrillarin-NoLS-RFP. DsRed-Nop56 was prepared by PCR-amplification from full-length mouse cDNA (clone IMAGE 5006404) and subcloning into the Sall/BamHI sites of pDsRed-m-C1. GFP-Nopp140 was obtained by PCR-amplification from rat cDNA kindly provided by T. Meier (Albert

Einstein College of Medicine of Yeshiva University, New York, USA) and subcloning into the EcoRI/BamHI sites of pEGFP-C2. P45 and MafG were PCR-amplified from mouse cDNAs kindly provided by V. Mignotte (Institut Cochin, Paris, France), and cloned into the EcoRI/BamHI sites of pEGFP-C1 and pDsRed-m-C1, respectively. DsRed-NoLS-MafG was constructed by inserting the entire NoLS DNA sequence into the XhoI/EcoRI sites of pDsRed-m-C1-MafG. GFP-Sirtuin 1 was obtained by PCR-amplification of the DNA sequence encoding human Sirtuin 1 from pBST-SIRT1 kindly provided by F. Ishikawa (Kyoto University, Kyoto, Japon) and subcloning into the Sall/BamHI sites of pEGFP-C1. The sequences of the oligonucleotides used for PCR-amplifications are available upon request.

Pull down experiments

For in vitro pull down assays, the GST, GST-NoLS and GST-Rev proteins were overexpressed in *E. coli* (BL21 DE3) and then induced 3 hours with 0.1 mM IPTG at 37°C. Lysates were obtained by enzymatic reaction with lysozyme (100 µg/ml) and DNase I (20 ng/µl) and clarified by ultracentrifugation at 65,000 g for 30 minutes. The clarified lysates were incubated with glutathione-sepharose beads (Amersham) for 2 hours at 4°C. GST fusion proteins bound to glutathione-sepharose beads were washed four times with wash buffer 1 (50 mM Tris-HCl pH 8, 1 M NaCl, 10% glycerol and complete) and once with binding buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2% NP40, 10% glycerol and complete). Complete corresponds to a cocktail of protease inhibitors (Roche). Proteins of HeLa cells transfected or not were extracted using lysis buffer (50 mM Tris-HCl pH 7.4, 500 mM NaCl, 1 mM EDTA, 1% NP40, 10% glycerol and complete). After centrifugation at 16,000 g for 15 minutes, the supernatants were adjusted to the binding buffer conditions and these whole cell extracts were incubated with fusion proteins bound to glutathione-sepharose beads. After

gentle shaking overnight at 4°C, the beads were centrifuged at 500 g for 2 minutes and washed four times in wash buffer 2 (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 1 mM EDTA, 0.2% NP40, 10% glycerol and complete). In experiments comparing NoLS and Rev affinities, the incubations were performed for times varying from 1.5 to 20 hours. Purified B23 was produced by digestion of GST-B23 overexpressed in *E. coli* with thrombin (1 unit for 30 μ l of beads) and purification on glutathione-sepharose beads as described above. For pull down assays performed with purified B23, the incubation time was 2 hours. In all cases, proteins corresponding to extracts recovered before and after pull down assays and proteins bound to beads were resuspended in SDS loading buffer, resolved by SDS-PAGE and analyzed by coomassie blue staining and immunoblotting.

Immunoblot analyses

Proteins resolved by 10% SDS-PAGE were transferred to nitrocellulose membranes and incubated with the following antibodies: rabbit polyclonal anti-B23 (kindly provided by M. O. J. Olson, University of Mississippi Medical Center, Jackson, USA), rabbit polyclonal anti-GFP (Roche), mouse monoclonal anti-nucleolin (Santa Cruz Biotechnology), and human autoimmune sera with specificities against Nop52 (C13; Savino et al., 1999) and Nopp140 (A10; characterized in the present study). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and the immunoreactivity was detected by chemiluminescence (Amersham) and quantified using MetaMorph® software (Universal Imaging Corp.) by measuring the average intensity of the bands.

References

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Figure legends

Fig. 1. A “synthetic” NoLS targets GFP to nucleoli. (A) Schematic representation of the generation of NoLS. The upper diagram corresponds to the DNA sequence encoding human Sirtuin 1, the middle one to the PCR product after misreading of the DNA polymerase and the bottom one to the resulting mutant protein. A partial nucleotide sequence of Sirtuin 1 is indicated in the upper diagram, and the corresponding misread sequence is indicated in the middle diagram. The resulting mutant protein corresponds to the first 87 amino acids of Sirtuin 1 (Δ Sirtuin 1) followed by a “synthetic” sequence of 93 amino acids (NoLS). The numbers are nucleotide or amino acid positions. (B) NoLS with the stretch of basic residues in red. (C) Constructs of NoLS containing the entire or partial basic stretch in red. (D) Fluorescence microscope observations of the NoLS constructs fused to GFP and expressed in HeLa cells. Only the constructs possessing the stretch of basic residues were predominantly (NoLS/31-63, i,j and NoLS/1-50, e,f), if not exclusively (NoLS, a,b and NoLS/33-93, c,d) localized in nucleoli whereas the other constructs were not (NoLS/51-93, k,l) or only slightly (NoLS/1-32, g,h) enriched in nucleoli. Arrowheads in panel b indicate nucleoli visible by phase-contrast image. Bar, 10 μ m.

Fig. 2. NoLS interacts directly with B23; this interaction involves the acidic stretches of B23. (A) GST and GST-NoLS pull down assays carried out using whole HeLa cell extracts. 1% of the extract used for each pull down assay (Input), 1% of the supernatant (S) and 1.5% of the proteins eluted from the bead pellet (P) after the indicated pull down assay were electrophoresed and immunoblotted to detect B23, Nopp140, Nucleolin and Nop52. B23 interacts specifically with GST-NoLS but not with GST. On the contrary, Nopp140, Nucleolin and Nop52 do not interact with GST-NoLS. (B) GST and GST-NoLS pull down

assays carried out using a purified recombinant B23. 2% of the supernatant (S) and 3% of the proteins eluted from the bead pellet (P) after the indicated pull down assay were electrophoresed and immunoblotted to detect B23. B23-NoLS interaction is direct. (C) Pull down assays performed on whole extracts prepared from HeLa cells transiently transfected with B23 wild-type (B23 wt) and B23 mutants fused to the C-terminus of GFP or DsRed as indicated. The mutants B23/121-186, B23/1-119, B23/1-132, B23/1-160, B23/1-186 and B23/187-294 correspond to the B23 mutants possessing the B23 residues 121-186, 1-119, 1-132, 1-160, 1-186 and 187-294, respectively. Black boxes on the schematic representations correspond to the stretches of acidic amino acids present in the B23 sequence. 1.5% of the extracts used for each pull down assay (Input) and 3% of the proteins eluted after the GST-NoLS pull down assay (GST-NoLS) were electrophoresed and immunoblotted using anti-B23 or anti-GFP antibodies. Only B23 mutants possessing at least one stretch of acidic amino acids interact with GST-NoLS. Asterisks indicate the endogenous B23 revealed only with the anti-B23 antibodies but not with the anti-GFP antibodies. Arrowheads point the overexpressed tagged-B23 or -mutant B23.

Fig. 3. NoLS possesses a stronger affinity for B23 than does HIV-1 Rev. Pull down experiments were performed using GST, GST-NoLS and GST-Rev on the same whole HeLa cell extract for increasing incubation times (1.5, 3 and 20 hours) and the binding of B23 was analyzed. (A) 1.5% of the proteins eluted after each pull down assay was electrophoresed and analyzed by coomassie blue staining. No significant loss of GST, GST-NoLS or GST-Rev occurred during the pull down assays. (B) 1.5% of the extract used for each pull down assay (Input) and 1.5% of the proteins eluted after the indicated pull down assay were electrophoresed and immunoblotted using anti-B23 antibodies. The signals corresponding to B23 were quantified and are expressed as a percentage of B23 present in the input. Contrary

to GST, both GST-NoLS and GST-Rev bound B23 but B23-NoLS interaction occurred more rapidly and more efficiently than B23-Rev interaction.

Fig. 4. Localization of GFP-NoLS in HeLa cells during the cell cycle. (A) HeLa cells were transfected with GFP-NoLS fusion and analyzed by fibrillarin or B23 immunolabeling (a,e), GFP-NoLS observation (b,f) and phase-contrast (d,h). The nucleolar localization of NoLS in interphasic cells was demonstrated using fibrillarin a marker of the DFC, and B23 preferentially localized to the GC of nucleoli. GFP-NoLS did not colocalize with fibrillarin as observed by comparing the enlargements (a-c, insets), whereas it colocalized with B23 as observed by comparing the enlargements (e-g, insets). (B) HeLa cells cotransfected with GFP-NoLS and DsRed-B23 were synchronized in mitosis. GFP-NoLS observations (a,e,i), DsRed-B23 observations (b,f,j) and superimpositions of both labelings in the same mitotic cells (c,g,k). The mitotic stage was determined by DAPI staining (d,h,l). GFP-NoLS colocalized with DsRed-B23 at the chromosome periphery in metaphase (a-d) and in numerous foci, the PNBs, in telophase (e-h) and early G1 (i-l). In early G1, GFP-NoLS and DsRed-B23 colocalized in the reformed nucleoli. These nucleoli are indicated by arrowheads (i-k). Bars, 10 μ m.

Fig. 5. NoLS still colocalizes with B23 after actinomycin D or roscovitine treatment and is delocalized from nucleoli to nucleoplasm when the endogenous B23 is selectively delocalized from nucleoli to nucleoplasm. (A) HeLa cells cotransfected with GFP-NoLS and mDsRed-B23 or with GFP-NoLS and fibrillarin-RFP were treated with actinomycin D (a-h), or roscovitine (i-p). After actinomycin D treatment, NoLS colocalized with B23 in the segregated nucleolus (a-d) but was excluded from the fibrillarin-containing caps (e-h). After roscovitine treatment, NoLS colocalized with B23 in large nuclear bodies (i-l) but not with

fibrillarin (m-p). (B) NIH-3T3 cells were transfected with GFP-B23-NES and analyzed by B23 immunolabeling (a-c) or cotransfected with GFP-B23-NES and DsRed-NoLS (d-f). After leptomycin B treatment, GFP-B23-NES was located in the nucleus and mostly excluded from nucleoli (a, arrowheads). Anti-B23 antibodies, revealing both endogenous and mutated B23, labeled the nucleoplasm and only slightly the nucleoli of GFP-B23-NES-expressing cells contrary to what was observed in cells not expressing GFP-B23-NES in which B23 labeling was mostly restricted to nucleoli (b). Thus, the endogenous B23 was mostly excluded from nucleoli in NIH-3T3 cells expressing GFP-B23-NES. In cells expressing GFP-B23-NES (d), DsRed-NoLS (e) was also mostly delocalized from nucleoli to nucleoplasm. Arrowheads point to nucleoli. Bars, 10 μ m.

Fig. 6. Fusion of NoLS to fibrillarin makes it possible to delocalize fibrillarin from the DFC to the GC of nucleoli; this mislocalization of fibrillarin in the GC of nucleoli induces the mislocalization of the fibrillarin partner Nop56 in the GC but does not affect the localization of Nopp140. HeLa cells were cotransfected with fibrillarin-GFP and DsRed-B23 (a-d), with fibrillarin-NoLS-GFP and DsRed-B23 (e-h), with fibrillarin-GFP and DsRed-Nop56 (i-l), with fibrillarin-NoLS-GFP and DsRed-Nop56 (m-p), with fibrillarin-RFP and GFP-Nopp140 (q-t), or with fibrillarin-NoLS-RFP and GFP-Nopp140 (u-x). Fibrillarin (a) localized in the DFC of nucleoli contrary to B23 (b) that localized preferentially in the GC as evidenced by the superimposition of both labelings (c) and by the comparison with the phase contrast image (d). When fused to NoLS, fibrillarin (e) was delocalized from the DFC to the GC of nucleoli and therefore colocalized with B23 (f) as evidenced by the superimposition of both labelings (g). Fibrillarin (i) colocalized with Nop56 (j) in the DFC of nucleoli as evidenced by the superimposition of both labelings (k). The delocalization of fibrillarin in the GC of nucleoli by the fusion of the NoLS (m) similarly modified the nucleolar localization of Nop56 (n), and

therefore both proteins still colocalized as demonstrated by the superimposition of both labelings (o). Fibrillarin (q) and Nopp140 (r) colocalized in the DFC of nucleoli as evidenced by the superimposition of both labelings (s). Contrary to Nop56, the delocalization of fibrillarin fused to NoLS (u) did not modify the nucleolar localization of Nopp140 (v) as shown by the superimposition of both labelings (w). Bar, 10 μ m.

Fig. 7. The fusion of NoLS to MafG makes it possible to delocalize the nuclear complex MafG-P45 to nucleoli. HeLa cells were cotransfected with GFP-P45 and DsRed-B23 (a-d), with DsRed-MafG and GFP-B23 (e-h), with DsRed-NoLS-MafG and GFP-B23 (i-l), with DsRed-NoLS-MafG and GFP-P45 (m-p), with DsRed-NoLS and GFP-P45 (q-t) or with DsRed-NoLS-MafG and GFP-Sirtuin 1 (u-x). P45 (a) and MafG (e) were localized in the nucleus and excluded from the nucleoli as observed by comparing with B23 localization (compare a,b and e,f) and by the superimpositions of labelings (c,g). When fused to NoLS, MafG (i) colocalized with B23 (j) in nucleoli as evidenced by the superimposition of both labelings (k). Targeting of MafG to nucleoli by addition of the NoLS induced a clear relocalization of P45 to the nucleoli (compare m, n, o and the phase contrast image p). Coexpression of DsRed-NoLS and GFP-P45 (q-t) showed that the expression of NoLS did not modify the nuclear localization of P45. Contrary to P45, targeting of MafG to nucleoli by the addition of NoLS (u) did not modify the nuclear localization of Sirtuin 1 (v). Bar, 10 μ m.

Fig. 1

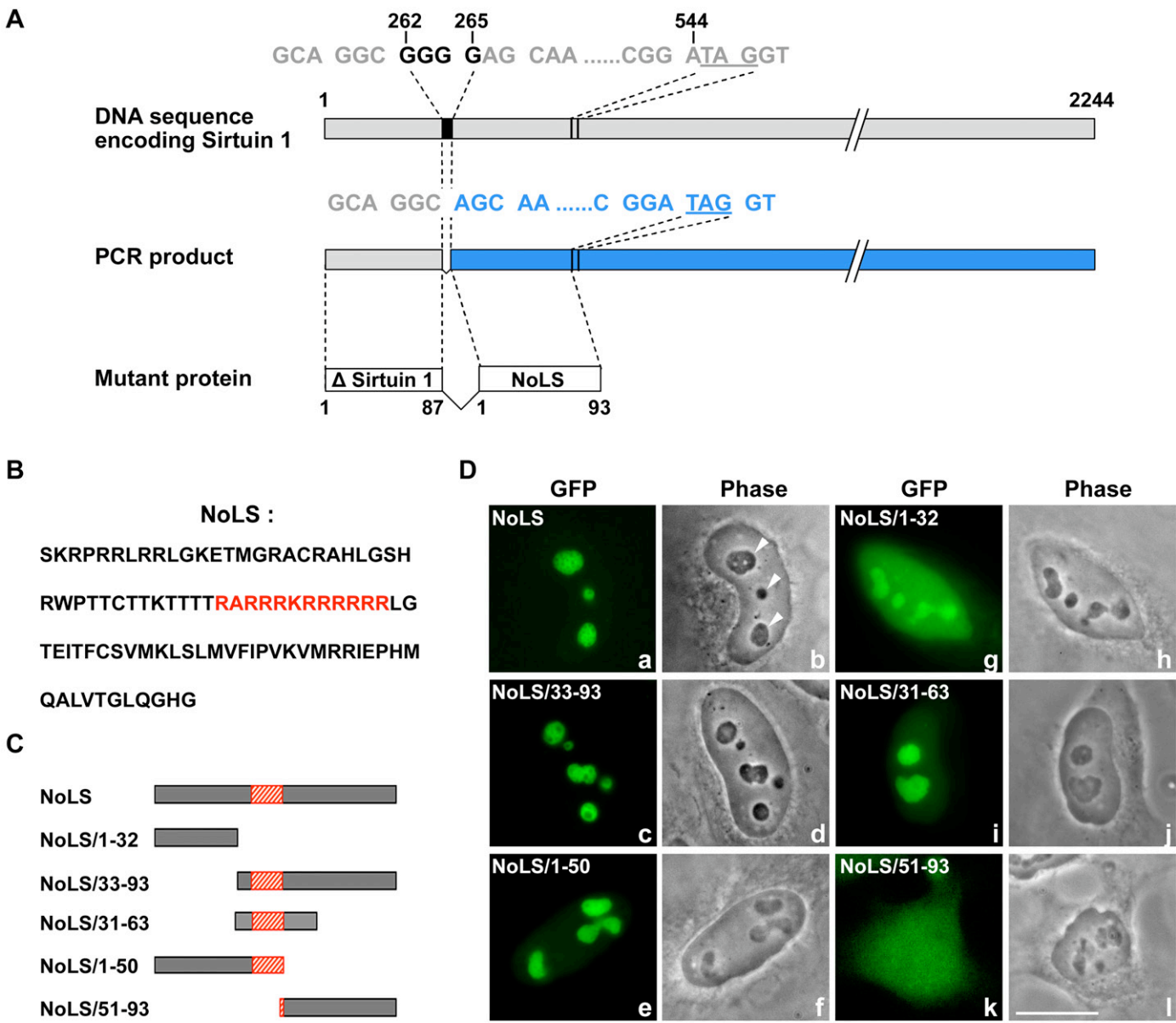


Fig. 2

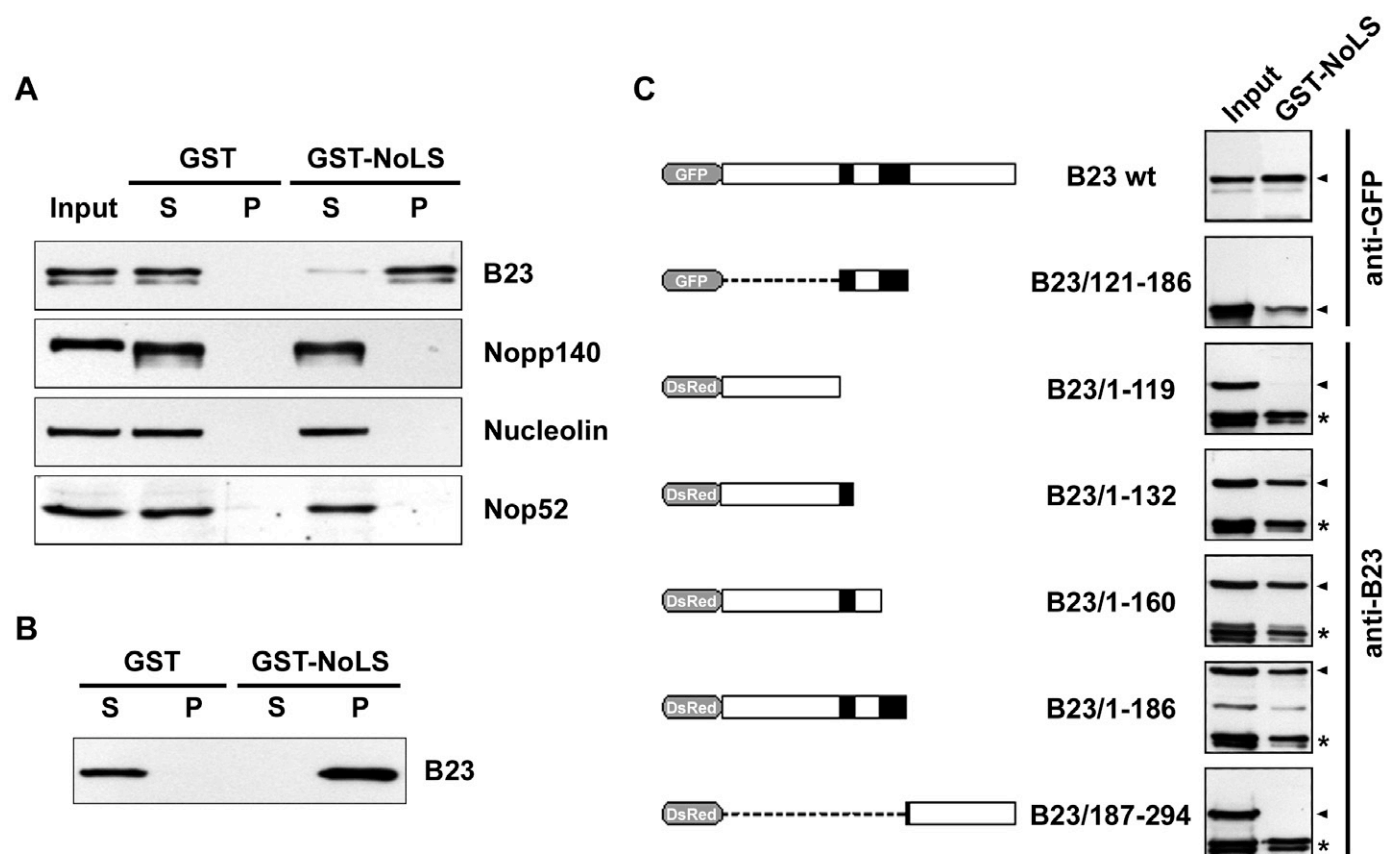


Fig. 3

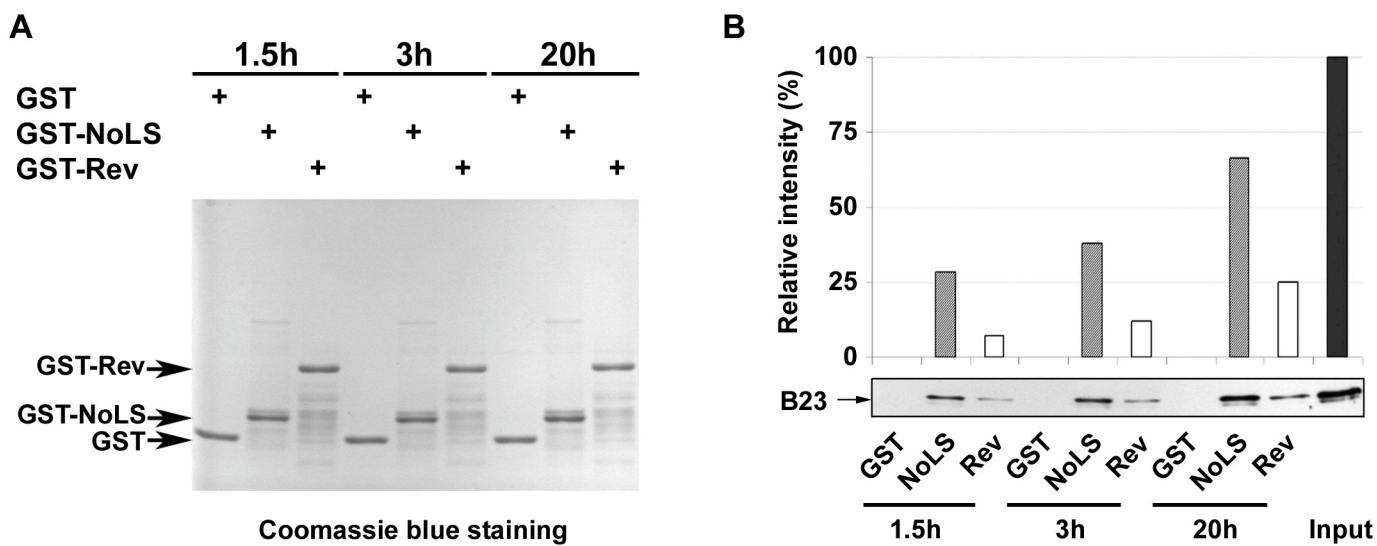


Fig. 4

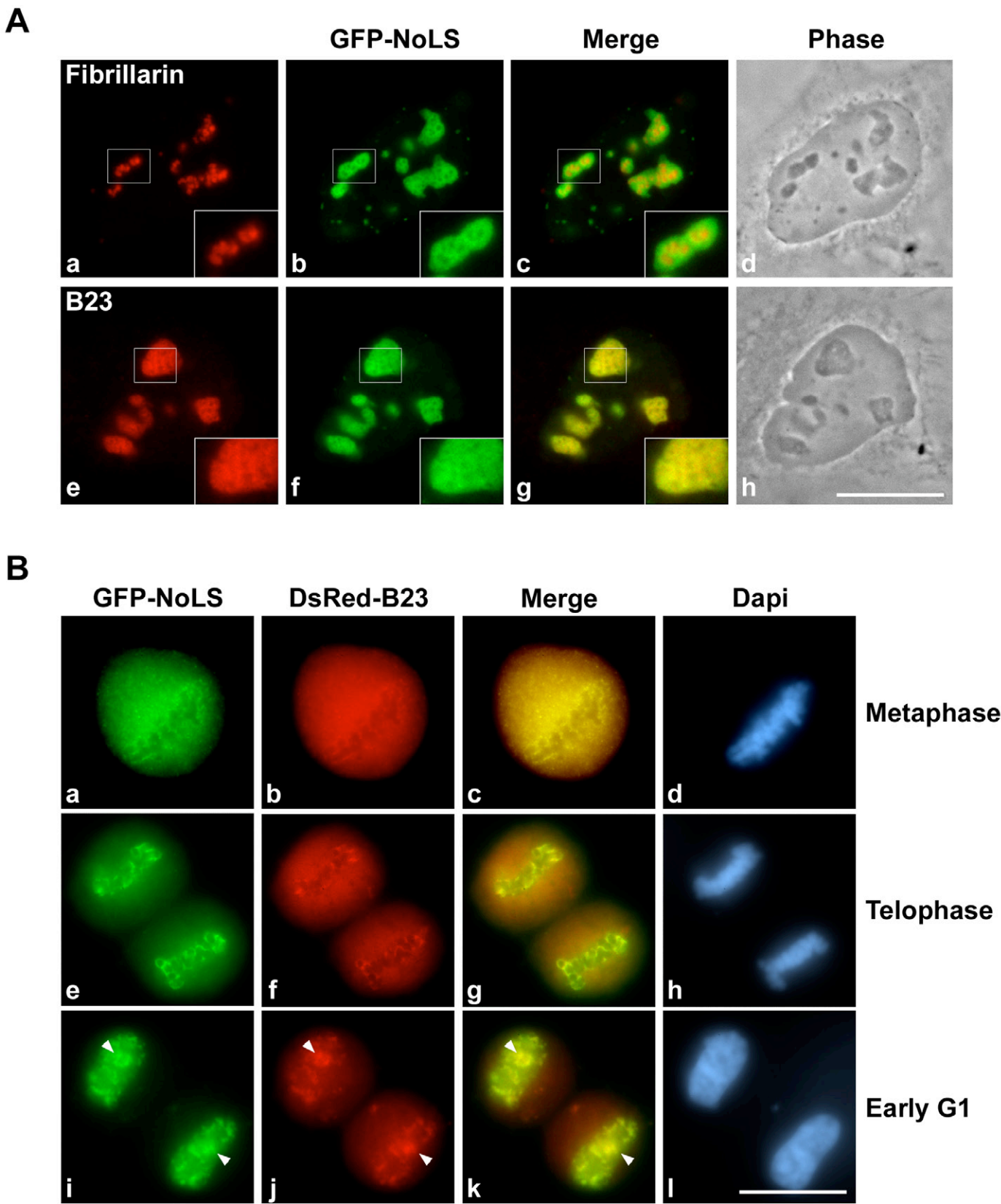
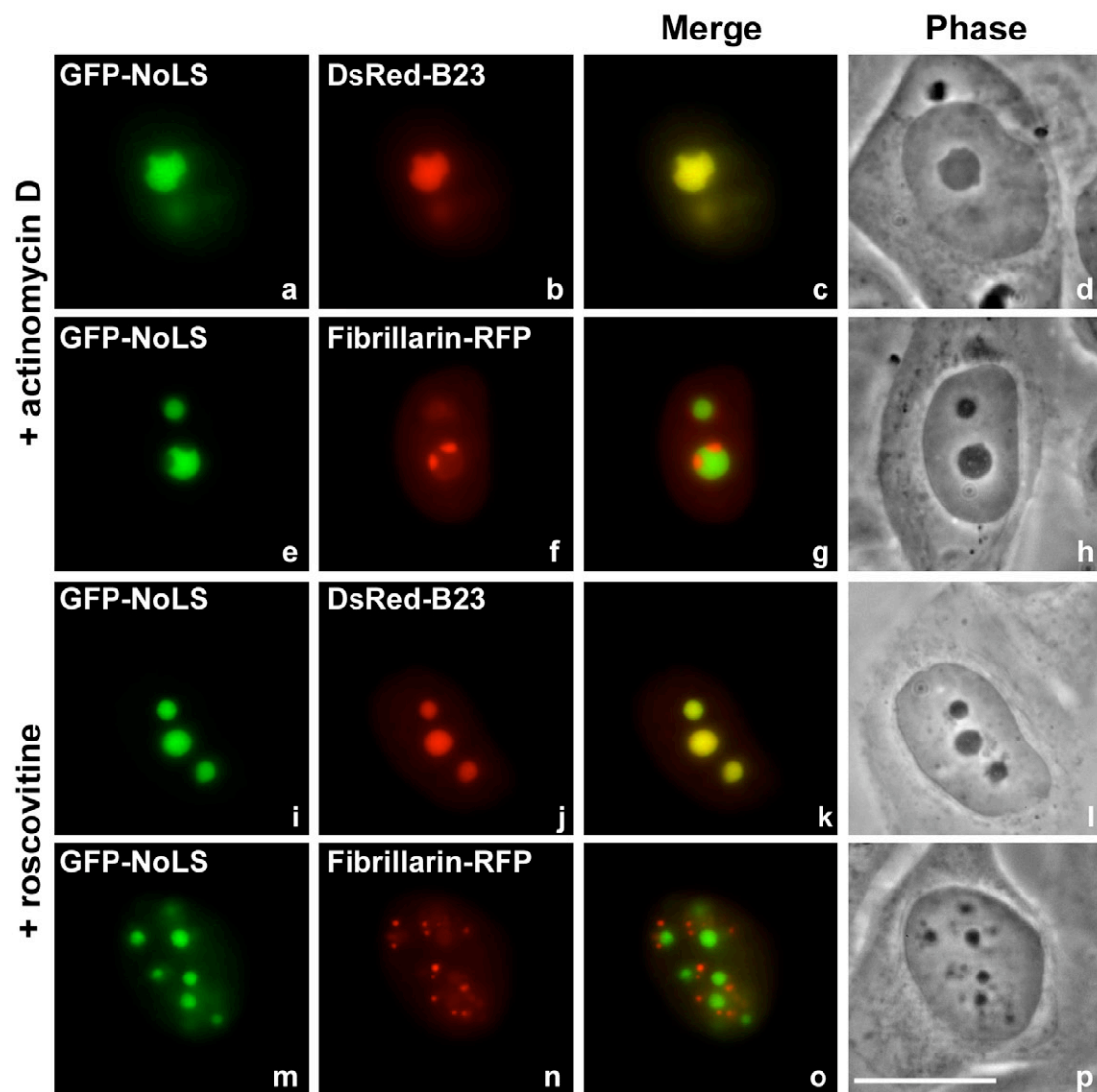


Fig. 5

A



B

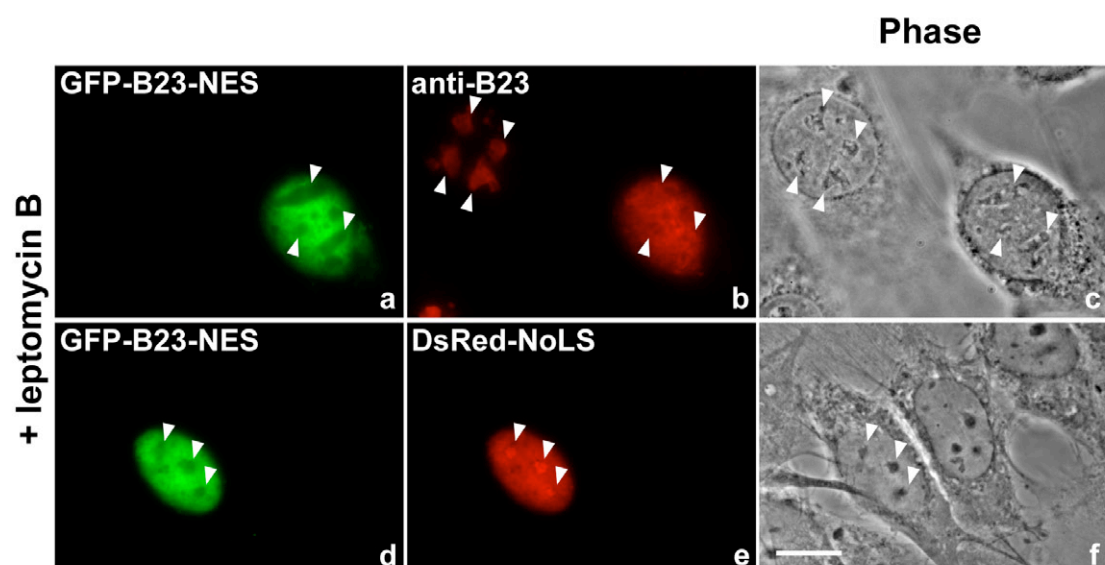


Fig. 6

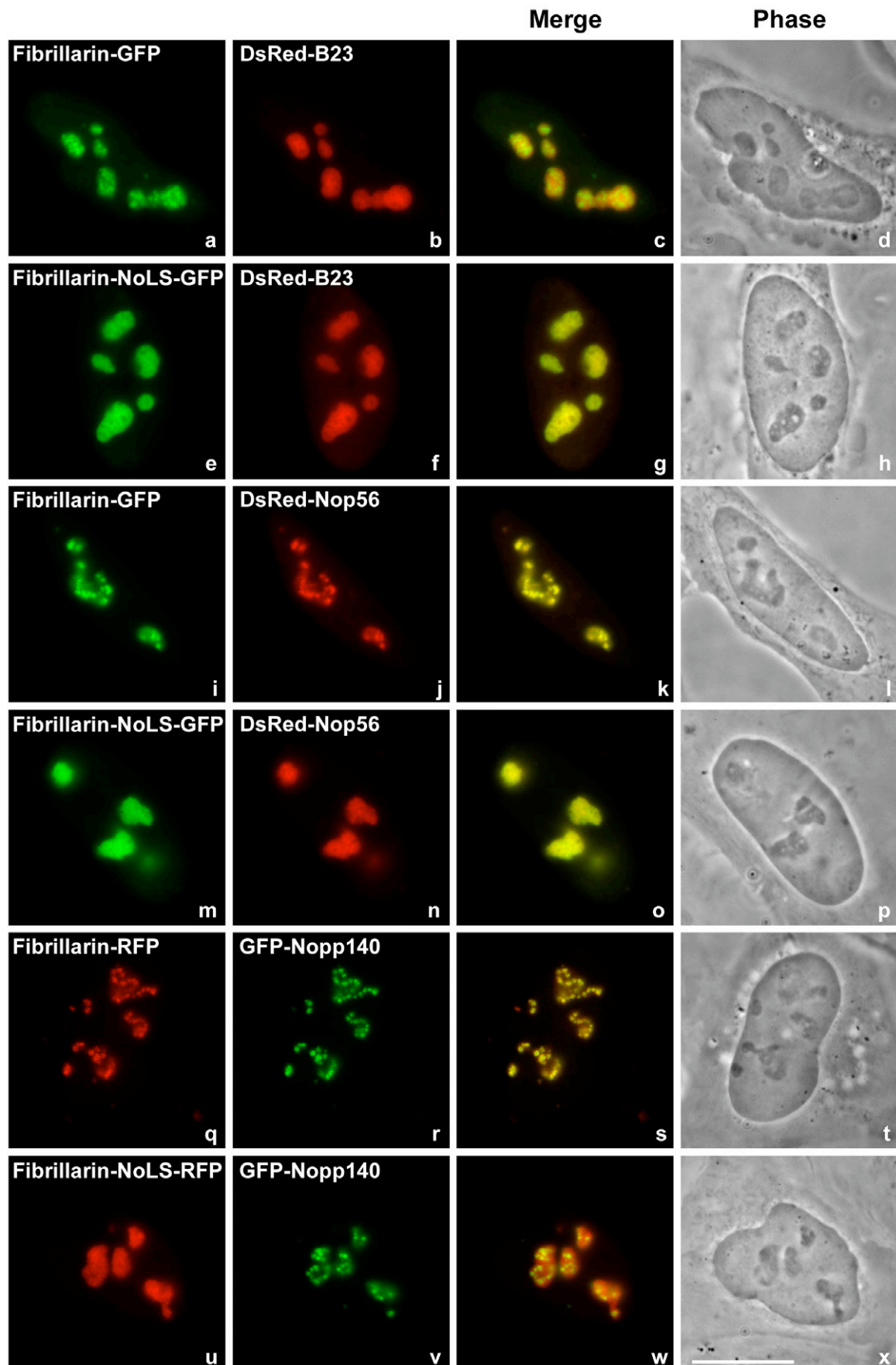


Fig. 7

